# Hedgehog Signaling in Duchenne Muscular Dystrophy

Research Thesis

Presented in partial fulfillment of the requirements for graduation *with Research Distinction* in Biological Sciences in the undergraduate colleges of The Ohio State University

by

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## Abstract

Duchenne muscular dystrophy (DMD) is a severe and lethal early childhood muscle disorder. It is due to genetic mutations in the *DMD* gene that disrupt dystrophin protein expression in all striated muscles. As a result of loss of dystrophin, skeletal muscles undergo cycles of degeneration and chronic inflammation. Skeletal muscle normally has the ability to regenerate and repair itself. However, in DMD, muscle regeneration progressively fails leading to a loss of muscle mass and hence, muscle function. The mechanisms involved in this progressive failure of muscle regeneration are not fully understood. However, they appear tightly linked to a parallel progressive increase in fibrotic tissue that gradually replaces functional muscle tissue. Understanding the molecular mechanisms that lead to the progressive loss of muscle function is essential because it can reveal new treatment opportunities to halt disease progression and prolong lifespan in these patients.

Towards this goal, our laboratory has been studying the effects of the muscle environment on the fate choices of interstitial muscle stem cells. These stem cells have the unique ability to differentiate into muscle thus contributing to regeneration, or into fibrotic cells, thus potentially shifting the balance toward fibrosis. We have previously shown that the dystrophic muscle environment blocks the myogenic differentiation of interstitial stem cells and instead promotes their differentiation into fibrotic lineages (Penton et al., *PLoS ONE*, 2013). We discovered that myogenic differentiation of interstitial stem cells is dependent upon active Hedgehog signalling and that the dystrophic muscle environment inhibits Hedgehog signalling in these stem cells. Because Hedgehog signalling has not been studied in muscle disease, this thesis focuses on defining the broader role of this signalling pathway in disease progression in DMD, using the well-established *mdx* mouse model of DMD. We treated dystrophic *mdx* mice *in vivo* with a specific Hedgehog activator in order to restore Hedgehog signalling in dystrophic muscles to wild type levels. Short term treatment (1 week) significantly improved muscle regeneration. Interestingly, we found that inflammation was decreased along-side the regenerative effects. Long-term treatment (2 months) of *mdx* mice with the Hedgehog agonist lead to a decrease in fibrosis and a significant improvement in motor function. We show for the first time that Hedgehog signalling is impaired in muscular dystrophy and that this contributes to disease progression. Our results also indicate that sustained pharmacological activation of Hedgehog signalling to wild type levels has positive effects on regeneration, inflammation, fibrosis and motor function. Further research is needed to better understand the cellular and molecular mechanisms involved in Hedgehog signalling and to identify Hedgehog inhibitors present in dystrophic muscle. This may reveal new potential targets for therapeutic intervention in muscular dystrophy.

**Acknowledgements**

I would like to acknowledge those who have contributed to my project in a significant way. My advisor, Dr. Federica Montanaro whom I would like to thank for giving me the opportunity to work in her lab and the ability to complete my own project. Without her guidance this thesis would not be possible. Dr. Chris Penton, a member of

our lab that helped me gain many of the skills and knowledge that I utilized throughout my research. Nelson Salgado, Dr. Hong Wang, Madison Caja and Sarah Tuthill, members of our lab who helped contribute data to the larger question which my thesis is aiming to answer. Finally, the members of my committee, including Dr. Helen Chamberlin and Dr. Denis Guttridge for their time and input on my work.

## Contributions

In order to better detail the reasoning behind my thesis research and the path that led to my findings I must acknowledge work completed by members of our lab that will be mentioned throughout my thesis. The details of the findings will be listed later, however, I would like to acknowledge the work that contributed to my research. My thesis is a contribution to a larger question and study our lab is conducting. Specific acknowledgement will be made when the data has been contributed by a member of the lab.

## Introduction

Duchenne muscular dystrophy (DMD) is a lethal muscle disease that affects about 1 in every 4,087 live births (Mendell et al., 2012b). DMD is characterized by progressive muscle weakness and degeneration due to mutations in the *DMD* gene which produces the intracellular protein, dystrophin. This protein plays a critical role in signaling and structure within muscle (Lapidos et al., 2004). When the expression or function of dystrophin is impaired, the muscle fiber becomes more permeable which can lead to fiber necrosis. While increased damage is the primary outcome of loss of dystrophin, many secondary issues also contribute to muscle pathology (Williams and Bloch, 1999). When muscle is damaged, it has the amazing capacity to repair and regenerate itself. Three main components of muscle repair are altered in DMD which include a chronic presence of inflammation, the deposition of fibrotic tissue and impaired regeneration. These aspects are an important area of study in better understanding DMD.

## Patients with DMD

DMD is an X chromosome linked disease that mainly affects boys. The disease is usually not diagnosed until between two and five years of age. At a young age, changes in motor function are signaled by indicators such as difficulty in standing up from the ground or walking up stairs. While these deficits are noticed, DMD patients tend to reach most milestones in motor function until seven years of age (Wicklund, 2013). However, without intervention patients usually loose ambulation by age 12. In addition to loss of ambulation patients also have respiratory insufficiency and cardiac disease. Aside from these debilitating symptoms, patients with DMD are more likely to have cognitive impairment involving language, attention deficit hyperactivity disorder, autism spectrum disorder, or obsessive compulsive disorder (Hendriksen and Vles, 2008). Without intervention, death occurs around age 20 due to cardiac or respiratory disease. However, current intervention and research have helped extend the average life-span to 25 years with some patients living into their 30's (Wicklund, 2013).

## Current Research

Since the primary cause of DMD is the loss of the dystrophin protein, researchers are working to restore dystrophin function. Due to the size of the gene, 2.4Mb, repairing functionality is difficult. One approach to restore functionality includes exon skipping targeted at the pre-mRNA level. This approach allows the reading frame to be restored in case of deletion mutations, or leads to the removal of mutated exons containing premature stop codons that are very common in DMD (Mendell et al., 2012a). Exon skipping is a large area of focus as it has the potential to be applied to 83% of DMD patients. However, using exon skipping is difficult as different sequences for skipping would be required for individual patients (Fairclough et al., 2011). Another treatment approach is gene therapy, which uses clinically safe viral vectors to deliver a functional copy of dystrophin. Because the *DMD* gene is the largest in the human genome, a full replacement is not feasible. Therefore, gene therapy introduces smaller or micro versions of dystrophin that function the same as, or close to the whole gene. These approaches are important in targeting the disease but they require an adequate amount of muscle in the

patient to have a beneficial effect. DMD has many secondary characteristics that worsen the disease over time which can alter the effectiveness of these approaches. Since these characteristics such as inflammation, regeneration and fibrosis are important in maintaining muscle, they are also a target of current research. Improving the secondary effects of the disease can contribute to the improvement of the efficacy of the treatments mentioned above. Currently, treatments in human DMD patients target inflammation using glucocorticoid corticosteroids. While it does lengthen the time that a patient can be ambulatory this intervention does not significantly lengthen life span (Flanigan, 2014). Targeting both the functionality of dystrophin and the secondary characteristics of the disease are important in studying DMD and new or alternative approaches are an important focus in research.

## Muscle Damage

### Inflammatory Response in Muscle Injury

Dystrophin is essential to cell signaling and cell structure within skeletal muscle. Dystrophin is a part of the dystrophin glycoprotein complex (DGC) which helps to link the intracellular cytoskeleton to the extracellular matrix (ECM). When dystrophin is not produced in DMD patients, the DGC becomes functionally impaired. Because of this change in structure, the permeability of the sarcolemma, the cell membrane of a muscle fiber, is increased (Shin et al., 2013). Calcium is able to leak through the sarcolemma with greater ease which activates proteases and leads to a necrotic fiber (Zhou and Lu, 2010). As a result of these changes, DMD patients experience chronic muscle damage.

When a muscle fiber is damaged the first step of repair involves the recruitment of inflammatory cells to the damage site. The response is present in order to aid in cleaning away any unneeded or unwanted material as well as to help stabilize functions of the repair process. These cells are mainly myeloid cells. Within 2 hours of muscle damage, neutrophils appear in the damaged area. In order to clean out the damaged tissue, macrophages that are phagocytic are recruited to the damaged area. These are also known as M1 macrophages that are pro-inflammatory and express inducible nitric oxide

synthase (iNOS) to produce cytotoxic levels of nitric oxide. The primary function of M1 macrophages is to help clear debris in the damaged area. M1 macrophages are then switched to the M2 phenotype which is non-phagocytic. While the timing and cause of the switch in phenotype is not completely understood, it is thought that phagocytosis of debris can cause a switch in phenotype (Fadok et al., 1998). When M2 macrophages are activated, they can release anti-inflammatory cytokines that help to deactivate M1 macrophages (Tidball et al., 2014). M2 macrophages promote tissue repair and their activity is in part controlled by CD206, a mannose receptor. The activation and expression of these inflammatory cells is highly regulated in healthy muscle repair, but the process in DMD is perturbed because of the chronic nature of muscle damage. In dystrophic muscle there is a coexistence of areas of active degeneration and areas of regeneration within the same muscle. Therefore, in DMD there is a chronic presence of these inflammatory cells and a temporal overlap of signals that regulate the differentiation and activation of M1 and M2 macrophages. The persistent presence of inflammatory cells can turn the beneficial signaling into one that promotes muscle damage (Abdel-Salam et al., 2009), impairs regeneration (Villalta et al., 2009), and promotes fibrosis (Zhou et al., 2006).

## Regeneration

In order to generate new fibers, muscle stem cells, also known as satellite cells become activated. Satellite cells are mononuclear cells located under the basal lamina, a layer of the ECM, that surrounds myofibers (Pallafacchina et al., 2013). Once activated, satellite cells proliferate and can either self-renew or differentiate. When satellite cells go through this process it is regulated by different basic helix-loop-helix transcription factors, which include myogenic factor 5 (Myf5), myoblast determination protein (MyoD), myogenin, and muscle regulatory factor (MRF4) which are typically expressed in that order (Karalaki et al., 2009). These transcription factors are regulated by the transcription factors paired box 7 and 3 (Pax7 and Pax3). Both Pax3 and Pax7 bind to promoters of MyoD and Myf5 and regulate their expression in the muscle (Wang and Rudnicki, 2012). Pax3 is mainly expressed during embryonic muscle development so

most of the focus in DMD has been on Pax7. Pax7 helps regulate the fate of satellite cells during proliferation and is down regulated during differentiation (Wang and Rudnicki, 2012). During satellite cell proliferation, macrophages are present in the damaged area. The inflammatory response is meant to be beneficial in repairing the muscle, but in DMD the response can sometimes be damaging. This is in part because different macrophages are a source of cytokines that can affect satellite cells (Hirata et al., 2003); for example, they can delay their myogenic differentiation (Merly et al., 1999). This is supported by *in vitro* experiments where the presence of M1 macrophages increases satellite cell proliferation while decreasing their myogenic differentiation. By contrast, M2 macrophages increase satellite cell differentiation (Arnold et al., 2007). This suggests that alterations in the inflammatory response and presence or absence of different macrophages can affect muscle regeneration. When proper satellite cell differentiation does not occur, myofiber regeneration is impaired which can give rise to the formation of split or branched fibers.

**Branched Fibers**

The fusion of mononuclear satellite cells with each other or with myofibers is what helps to form new multinucleated myofibers or regenerate damaged segments of pre-existing fibers. When a muscle fiber or a segment of a muscle fiber degenerates, the basal lamina is left behind and is used as a scaffold (Turner and Badylak, 2012). Satellite cells proliferate, enter this tube of basal lamina, and begin to fuse with each other. The cells create multiple small-diameter immature myotubes spanning the length of the degenerated myofiber segment. In a normal regenerative process, these myotubes increase in diameter as satellite cells continue to differentiate and fuse with them, thus, increasing their size. Once the myotubes touch each other and have filled the space originally occupied by the myofiber, they fuse with each other and, thus, reconstitute a single continuous myofiber. However, in DMD, satellite cell differentiation is impaired and myotubes can fail to fuse into one large myofiber within the matrix scaffold. When this fusion fails to occur, the individual myotubes stop growing and begin to lay down their own individual basal lamina. This allows them to be mechanically more stable and

to complete maturation. However, this blocks myotube fusion leading to the formation of fibers that are branched (Ciciliot and Schiaffino, 2010). The fibers can branch into two, or more individual fibers depending on when the myotubes stop growing and fusing with each other **(Figure 1).**

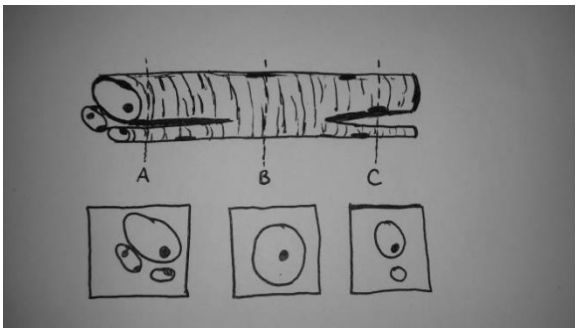



**Figure 1.** Drawing of a branched fiber and splitting points in serial sections. Cross sections A, B and C show the structure of the single fiber at the branching points.

Branched fibers are weaker at branching points and consequently, do not function as effectively as one continuous fiber. In contrast to non-branched fibers, branched fibers have been shown to have reduced isometric force, have altered contraction times (Chan and Head, 2011), and be more prone to contraction-induced damage (Chan et al., 2007). When there is an increased chance for damage, the fibers are more susceptible to necrosis which contributes to the chronic cycle of degeneration and regeneration.

## Fibrosis

An important part of muscle structure is the extracellular matrix (ECM) which helps to provide a scaffold for the muscle. Existing within skeletal muscle are fibroblasts, cells that synthesize ECM surrounding muscle. Fibroblasts play an important role during regeneration by stimulating the secretion of necessary ECM precursors, including proteins such as collagen, to promote structural scaffolding. The production of substances needed in the ECM, such as collagens, is tightly regulated in muscle repair (Murphy et al., 2011). However, as occurs in DMD patients, the impairment of this process can lead to excessive deposition of fibrous tissue which can then damage the structure and function of muscle. Different growth factors which are altered in the dystrophic environment can promote deposition of fibrotic material such as collagen I. Dystrophic

muscle has a different micro environment compared to healthy muscle. This environment can cause macrophages to promote fibrosis and is thought to promote the conversion of satellite cells to a fibrogenic lineage (Brack and Rando, 2007). A main growth factor that is altered in the dystrophic environment is transforming growth factor beta (TGF-β). This protein can be secreted from cells such as macrophages and alters the regeneration process in muscle. TGF-β can increase fibroblast proliferation and cause increased production of collagen (Smith et al., 2007). All of these alterations in DMD promote fibrosis in the muscle and reduce the functional capacity of the muscle. Researchers are working to target not only fibrosis, but many of the aspects mentioned above in an effort to improve the structure and function of dystrophic muscle.

As a result of the altered and out of sync regeneration and degeneration in DMD, research is focused on obtaining a better understanding of the secondary processes involved in order to target these processes in the disease. Finding alternative pathways which affect the disease is very crucial to understand the disease as a whole and provide alternative approaches to treatments and research. One such pathway which is not fully understood in terms of its function and purpose in adult muscle is the Hedgehog pathway.

## The Hedgehog Pathway

Our laboratory has been studying how the muscle environment affects the differentiation of interstitial muscle stem cells towards either a myogenic or fibrotic state. Interstitial stem cells are distinct from satellite cells and primarily reside in the connective tissue in-between myofibers. Members of our lab have previously shown that myogenic differentiation of interstitial stem cells is completely blocked in the dystrophic environment where these cells appear to instead differentiate into fibroblasts (Penton et al., 2013). With the goal of targeting pathways that alter the fate of these stem cells, our lab used RT-PCR to identify signaling pathways that are altered in interstitial stem cells isolated from muscles of dystrophic *mdx* mice compared to wild type mice. We found that Hedgehog (Hh) signaling was inhibited in interstitial stem cells from dystrophic muscle. Because Hh signaling is important for myogenic fate determination during embryonic development, we hypothesized that Hh may similarly regulate the myogenic

differentiation of interstitial stem cells in adult muscle. When purmorphamine, an Hh agonist, was administered to interstitial stem cells isolated from dystrophic muscle, myogenesis was restored. This led us to further investigate what effects Hh signaling would have throughout dystrophic muscle. This was particularly important given a recent report that decreased Hh signaling in injured wild type muscle impairs regeneration and promotes fibrosis(Straface et al., 2009), an outcome similar to dystrophic muscle. The mechanisms and significance of the pathway are not well understood, which is why it is imperative to study its effects on muscle regeneration.

**History**

Hedghog  was first discovered in *drosophila melanogaster* in 1980 by Christiane Nüsslein-Volhard and Eric Wieschaus(Nusslein-Volhard and Wieschaus, 1980). It was named Hh because the mutations caused a spike like appearance on the drosophila larvae which look similar to the spikes of a hedgehog. The pathway's activation is controlled by different ligands. Overtime, three ligands in mammals have been discovered: Sonic Hh, Indian Hh and Desert Hh. These ligands and the signaling pathway mainly contribute to embryonic development but altered activation in adults has been linked to different diseases such as cancer(Beachy et al., 2004) or fibrosis(Piccioni et al., 2014).

**Embryonic Developmental Significance**

The Hedgehog (Hh) pathway plays a crucial role in embryonic development. Each of the different ligands performs separate roles in embryogenesis. Sonic Hh is important in the polarization of the limb bud and notochord. Indian Hh is important in bone and cartilage development and Desert Hh is important in germ cell formation (Briscoe and Therond, 2013). If Hh is impaired during embryogenesis different developmental diseases can occur. Aside from the embryonic importance, Hh has been a focal point of research especially in relation to adult function such as in cancers (Ruch and Kim, 2013) and more recently, in muscle and its effects on fibrosis (Straface et al., 2009).

**Pathway Mechanism**

The three Hh ligands bind to a transmembrane protein receptor known as patched-1 (Ptch). Ptch is an inhibitor of the transmembrane protein smoothened (Smo). Upon ligand binding, Ptch is unable to inhibit Smo. Once Smo is activated, it can activate Gli2 and Gli3, two transcription factors, which then promote the transcription of Hh target genes including but not limited to Gli1, Hhip, and Ptch. Since Ptch is a product of the pathway it participates in a negative feedback cycle. A known Hh agonist, purmorphamine can activate the Hh pathway by directly binding and activating Smo. Therefore, purmorphamine bypasses the inhibition by Ptch and the need for Hh ligands. We used purmorphamine as a specific Hh agonist to study the effects of Hh activation in dystrophic *mdx* mice (**Figure 2)**.

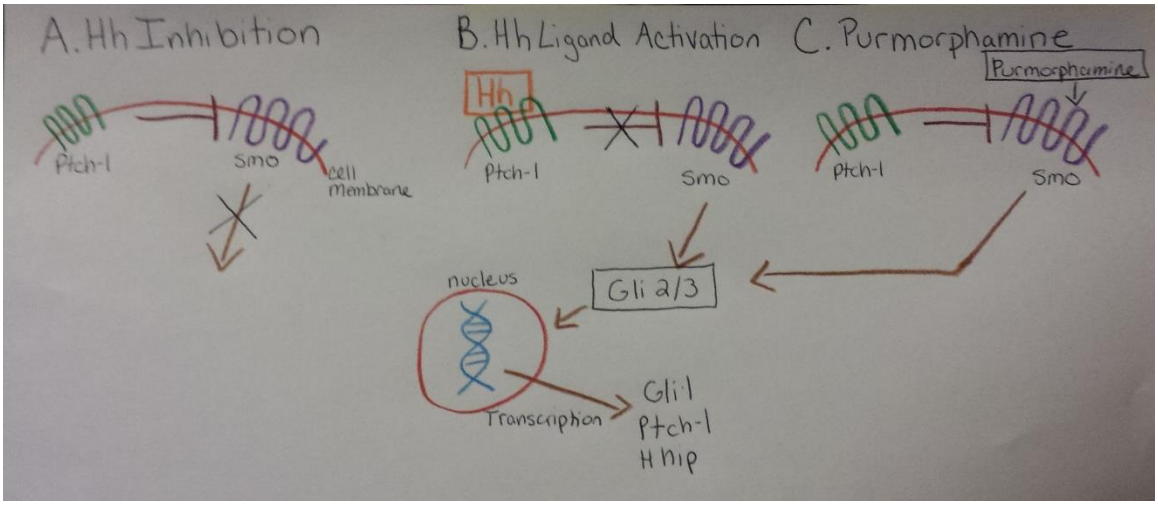



**Figure 2 A.** In the absence of ligands, Ptch blocks the activation of Smo and hence, the activation of the Hh pathway. **B.** When a Hh ligand binds to Ptch this stops the inhibition that Ptch has on Smo. When Smo is no longer blocked the pathway can become active. When active, the pathway transcribes different target genes via activation of the transcription factors Gli2 and Gli3. **C.** We have bypassed the need for Hh ligands and use the Hh agonist purmorphamine, to directly bind to and activate Smo and the pathway.

### Significance in post natal muscle

While the Hh pathway plays an important role in embryonic development, its activation and inhibition post natally is also important in the body. When the activity of the signaling pathway is unregulated in muscle, its activation or repression can have deleterious effects. The signaling of the pathway is noted to be located at the primary cilia of myogenic cells and that active Hh signaling promotes myogenic differentiation (Fu et al., 2014). Inhibition of Hh signaling has also been shown to decrease MyoD and Myf5 expression which alters satellite cell activation (Straface et al., 2009; Voronova et al., 2013). The regulation of the Hh pathway is not only important in dystrophic muscle as studied in my thesis but it is also important in regulating myogenesis and preventing fibrosis in healthy skeletal muscle (Straface et al., 2009).

## The *mdx* Mouse

While completing the study of DMD for this thesis, I have utilized the *mdx* mouse. This model was first reported in 1984 after a spontaneous mutation in C57BL/10 mice arose (Bulfield et al., 1984). The mutation is present in the *DMD* gene on the X chromosome in the mouse as it is in the human. While the phenotype in the *mdx* mouse is not as severe as in DMD patients, it can give a good indication of potential targets and therapies in research. The mice are different from patients with DMD in that while they show histopathology similar to humans, they remain ambulant throughout their life span. The general progression of the disease is also different than in humans. While in human patients there is a continual cycle of degeneration and damage in the muscle the mice have what can be referred to as a crisis period. During the first few weeks of life there is a continual cycle of degeneration and regeneration in parallel with inflammation. After this period the muscle begins to stabilize. Increased fibrosis is detectable by 12 weeks of age and remains relatively stable until about 9 months of age when it steadily increases concomitant with a decrease in myofiber regeneration. A layout of the disease progression in mice by their age can be seen in **Figure 3**. One known exception to the disease progression in *mdx* mice is the diaphragm. This muscle continues to have cycles

of degeneration and regeneration and does not stabilize as other skeletal muscles do. This makes the diaphragm a model that is more similar to the pathology seen in humans.

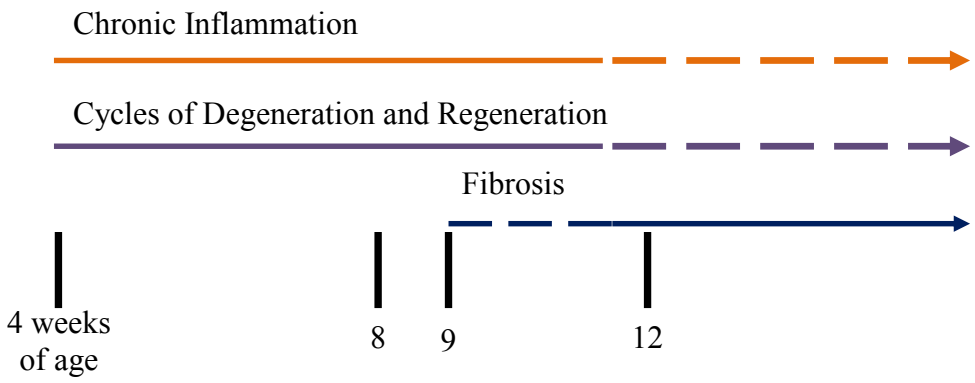



**Figure 3.** In limb muscle, starting around 3 weeks of age and peaking around 4 weeks, there is chronic inflammation that runs in parallel with the degeneration and regeneration. Once the 'crisis' period ends between 9 and 12 weeks of age the disease stabilizes with detectable levels of increased fibrosis which remain constant until about 9 months. After 9 months, fibrosis increases continuously and muscle degeneration and inflammation also increase continuously.

## Focus of this thesis

While there has been a lot of research in understanding DMD, there is much that is still unknown. It is important to understand the mechanisms and pathways involved in the secondary aspects of the disease. Our lab was working to answer a larger question of the effects of Hh inhibition in *mdx* mice and the long-term effects on fibrosis deposition. To answer this question, members of the lab looked at fibrosis deposition after activating Hh signaling in *mdx* mice with purmorphamine for two months. They found that inducing Hh signaling significantly decreased the amount of fibrosis in the diaphragm of *mdx* mice along with other pathological characteristics. My research project involved the characterization of *mdx* mice treated with Purmorphamine before the onset of detectable fibrosis, in order to gain a better understanding of how Hh signaling modifies fibrosis in *mdx* mice. This thesis not only focuses on the effects of Hh signaling during a chronic period of degeneration and regeneration, but also reports on interesting differences in the

response of limb (quadriceps) and diaphragm muscles to Hh activation. While both muscle types are frequently studied, the diaphragm is noted as a closer representation of human DMD as it is more severely affected by the disease than the limb muscle (Dupont-Versteegden and McCarter, 1992; Stedman et al., 1991). Having an effect on the diaphragm may give a better indication of the impact of Hh signaling in human dystrophic muscle.

The initial cause of DMD is the loss of dystrophin which damages the integrity of the muscle fiber. Currently, scientists are aiming to restore, or partially restore dystrophin as a therapy for the disease. Research has also shown that many of the outcomes of the disease are caused by secondary responses in the muscle environment. Understanding these processes may help scientists find alternate treatments in order to lessen the characteristics of the disease. The objective of this study is not to try and restore dystrophin but rather to study different features that may alter the disease. The effects of Hh signaling will open up different approaches to research and provide insight into potential treatment targets.

## Methods

**Mice:** Dystrophic mice involved in this experiment are m*dx5cv* mice originally obtained from Jackson Laboratories (B6Ros.Cg-*Dmd*$^{mdx\text{-}5Cv}$/J) and bred in-house. Wild-type (WT) mice also originated from Jackson Laboratories (C57BL/6J) and have been bred in-house. Combinations of male and female mice were used for the experiment and are not distinguished in separate categories. Mice were kept in similar environments throughout the experiment and fed a low fat diet. All procedures involved in this study followed protocols approved by the Institutional Animal Care and Use Committee at Nationwide Children's Hospital.

**Purmorphamine injections:** *Mdx5cv* mice were injected intraperitoneally (IP) with purmorphamine, an Hh agonist (100ul/10mg) or vehicle solution every 12 hours using 1mL sub Q 26 5/8 syringes from BD Biosciences. Purmorphamine was diluted in a vehicle solution composed of 10% DMSO, 18% Chremophore and 25% glucose in

distilled water. Using this protocol of purmorphamine injections we are able to restore the Hh activity back up to WT levels in *mdx* mice.

**Histology:** Dissected diaphragm and quadriceps muscle were mounted in trigacanth gum and frozen in liquid nitrogen. Tissues were cryosectioned at 10µm.

**Hematoxylin and Eosin:** Tissues were stained with Gills-3 Hematoxylin and Eosin-Y. Tissues were stained in Hematoxylin for 6 minutes then rinsed in warm water followed by a rinse in a 2% Ammonium Hydroxide in water solution followed by a rinse in water. Tissues were then dehydrated through a series of ethanol rinses; 30%, 95%, and 100% then placed in Xylene and mounted.

**Immunohistochemistry:** Tissue sections were fixed according to the primary antibody being used. For staining for embryonic myosin heavy chain (eMHC) and Pax7, sections were fixed in 4% paraformaldehyde in PBS for 15 minutes. For staining for CD45, sections were fixed in acetone at -20 degrees Celsius for 2 minutes then allowed to dry completely. All fixed tissues were then washed multiple times in PBS and blocked for 1 hour at room temperature in 1xPBS, 5% Horse serum and 2% BSA. Next, tissues were incubated overnight in the primary antibody solution at 4°C. Following primary incubation, sections were incubated for 1 hour at room temperature with the secondary antibody in block. Finally, the tissues were stained with DAPI and mounted with NPG mounting medium.

For Pax7 staining, an antigen retrieval step in 10mM Sodium Citrate Solution for 30 minutes at 100 degrees Celsius was done after fixation in 4% paraformaldehyde.

For primary antibodies made in mouse (see table), an additional Fab Fragment incubation step was included after the 1 hour blocking step above. Fab Fragment at 0.1mg/mL was added to the tissues for 2 hours at room temperature. The tissues then went through a post fixation in 0.1% paraformaldehyde for 5 minutes before incubation with the primary.

| Antibody | Species | Dilution | Reference |
|---|---|---|---|
| eMHC | Mouse | 1:10 | DSHB F1.652 |

| | | | |
|---|---|---|---|
| Pax7 | Mouse | 1:100 | *See below |
| CD45 | Rat | 1:200 | BD Biosciences 14-0451-81 |
| Laminin | Rat | 1:400 | Sigma L-9393 |
| Alexa 488 Donkey anti-Rat | Donkey | 1:200 | Jackson 712-585-150 |
| Rhodamine Red Donkey anti-Rat | Donkey | 1:200 | Jackson 712-295-150 |
| Alexa 488 Donkey anti-Mouse | Donkey | 1:200 | Jackson 712-545-153 |
| Fab Fragment Donkey anti-Mouse IgG | Donkey | 0.1mg/mL | Jackson 715-007-003 |

*Pax7 kindly provided by Denis Guttridge, Ohio State College of Medicine, Columbus, OH

**Statistical Analysis**

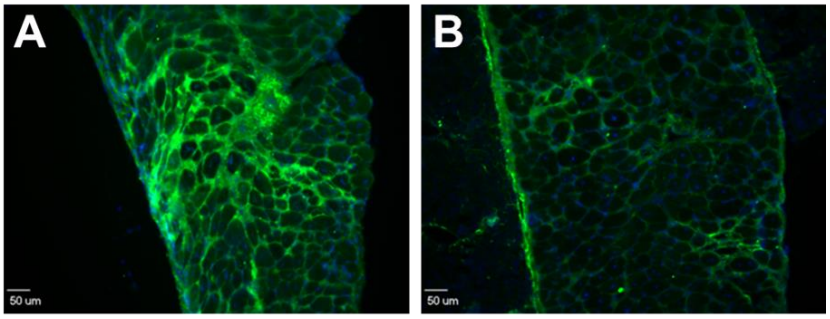
Photographs were taken at 20x magnification. All analyses were done on montages of the entire section of muscle created using Slidebook software. Images were taken at the same exposure. Staining for eMHC, and Pax7 were quantified using visual representation of fibers and all parameters quantified were normalized to the total number of fibers in the tissue section. Staining for CD45 was done comparing the total area of the tissue to the total area of CD45 positive stained sections. When area was determined all images were adjusted to the same threshold value in ImageJ (NIH). Threshold values were adjusted for a few sections where the staining was visually uniformly dimmer. All analyses of tissues were done using ImageJ (NIH).

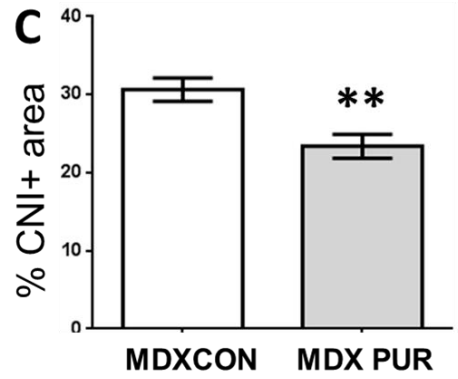
All image analysis between control mice and treated mice was done using a two-tailed unpaired Student's t-test. Significance was set at $P<0.05$.

**Rational For My Study**

Our lab began looking at Hh signalling when we found that it restored myogenesis in interstitial satellite cells. Since these cells took on a myogenic rather than fibrotic fate we hypothesized that restoring Hh activation in muscle may decrease fibrosis. In order to test this, members of our lab treated mice for 2 months, from 4 weeks of age to 12 weeks of age, and collected muscle tissue to test the pathological outcome in the mice. In order to see if the activation did alter fibrosis, Sarah Tuthill looked at fibrosis in diaphragm and quadriceps muscles using collagen I as a marker for fibrosis. She found that fibrosis was significantly reduced in the diaphragm, **Figure 4**.

**Figure 4. A.** Diaphragm at 12 weeks of age in a control *mdx* mouse treated with vehicle solution alone stained for collagen I (green) and Dapi (blue). **B.** Diaphragm of an *mdx* mouse treated for 2 months with Purmorphamine stained for collagen I and Dapi. **C.** *Mdx* mice treated for 2 months with purmorphamine show a significant decrease in collagen I deposition in the diaphragm. ** P<0.01. Control N=2, Treated N=3. Staining and quantifications done by Sarah Tuthill.




When fibrotic tissue is deposited it replaces muscle tissue. It would be inferred that if you are decreasing fibrosis then less muscle tissue would be lost. This led us to see if the amount of tissue gained or lost was the outcome of activating Hh signalling. In order to test the amount of muscle tissue present, we looked at the number

of fibers within a specified area of tissue. We used tissues stained with laminin and counted the number of fibers per square millimetre of tissue, **Figure 5.**

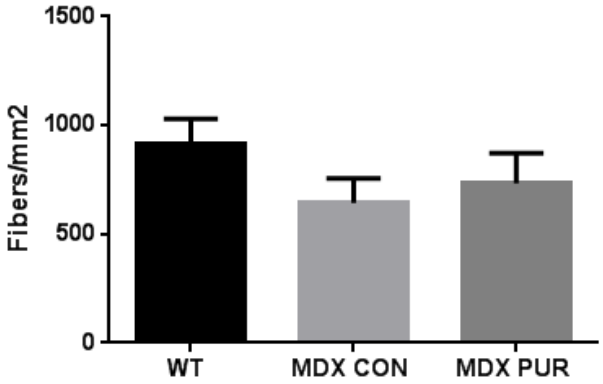



**Figure 5.** In diaphragm of mice treated for 2 months quantified for the total number of fibers per mm². WT N=5, Control N= 6, Treated N=3. Staining and quantifications contributed by Samantha Devenport and Dr. Hong Wang.

Although we saw no significant changes in muscle fibers per area in the diaphragm, there was a trend for an increased myofiber number in treated mice. We next asked if treatment improved regeneration. We quantified the percentage of myofibers with central nuclei, which denote a regenerated fiber, as a measure for regeneration in the tissue. We found that in the diaphragm of treated *mdx* mice there was a significant reduction in myofibers with central nuclei, denoting less fibers that have gone through regeneration, **Figure 6**. From this we could infer one of two things. Either, treatment is impairing regeneration, because there are less fibers being regenerated or, we are improving the structure of the muscle to the point where less fibers need to go through regeneration. To address this question we had to look at earlier time points to determine whether activation of Hh signalling by purmorphamine altered one or both key modulators of fibrosis: satellite cell function and inflammation. Therefore, in this thesis I am reporting the characterization of *mdx* mice at the end of a short pulse treatment with Purmorphamine from 8 to 9 weeks of age, before the onset of fibrosis.

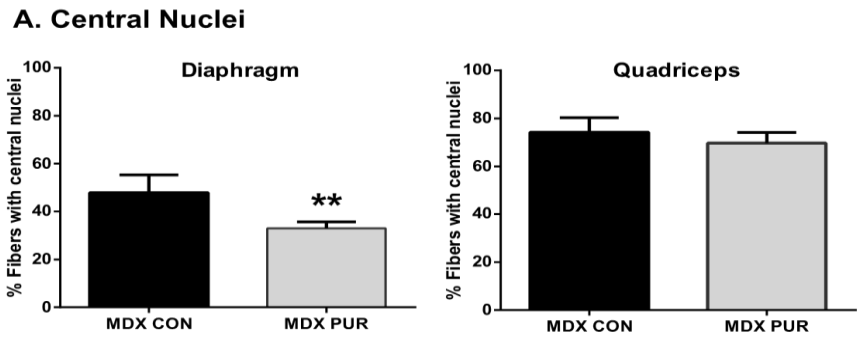


**Figure 6. A.** Central Nuclei values compared to the total number of fibers in entire tissue sections of diaphragm and quadriceps in 2 month control and treated mice. ** P<0.01. Staining and quantifications contributed by Samantha Devenport and Dr. Hong Wang.

## Results

Initial studies in the lab focused on the effects of long-term activation of Hh signaling from 4 to 12 weeks of age in *mdx* mice. These studies revealed a selective inhibition of fibrosis in the diaphragm. To better understand how the activation inhibited fibrosis, I performed short term, 1 week, treatments at 8 weeks of age, before the onset of fibrosis. We administered purmorphamine, a hedgehog agonist, IP twice a day in twelve hour intervals at a concentration for 100ul/10g body mass starting at 8 weeks of age. Control mice also received an injection of vehicle solution. After one week of treatment tissues were collected.

### General Inflammation

Since I was aiming to better understand the effects seen after two months of treatment in the mice, I first looked at inflammation as it contributes to fibrosis in *mdx* mice. To first look at inflammation I used a general marker for immune cells, CD45, a marker for Hematopoietic cells which includes all blood lineages, with the exception of red blood cells, to measure inflammation. The mice treated with purmorphamine were found to have a significant reduction in the inflammatory response in diaphragms compared to controls, however, the same results were not seen in the quadriceps, **Figure 7 A and B**. Interestingly, the diaphragm of control *mdx* mice showed large areas of CD45 staining while treated mice primarily showed small groups of CD45 positive cells interspersed between fibers. When comparing the inflammation to the general pathology of the tissue in controls versus treated mice there are stark differences. In areas where there is inflammation in control mice, the tissue has large patches of degeneration and regeneration seen throughout the tissue. These large areas of tissue remodeling are not seen in the treated mice. Rather, there are only small areas with some interstitial thickening and increased numbers of mononuclear cells, **Figure 7 C and D.** I carried out a similar analysis on mice treated for 2 months to determine whether this decrease in

inflammation was sustained. I found that here was a slight decrease in inflammation but nothing significant in the diaphragm, **Figure 8**.

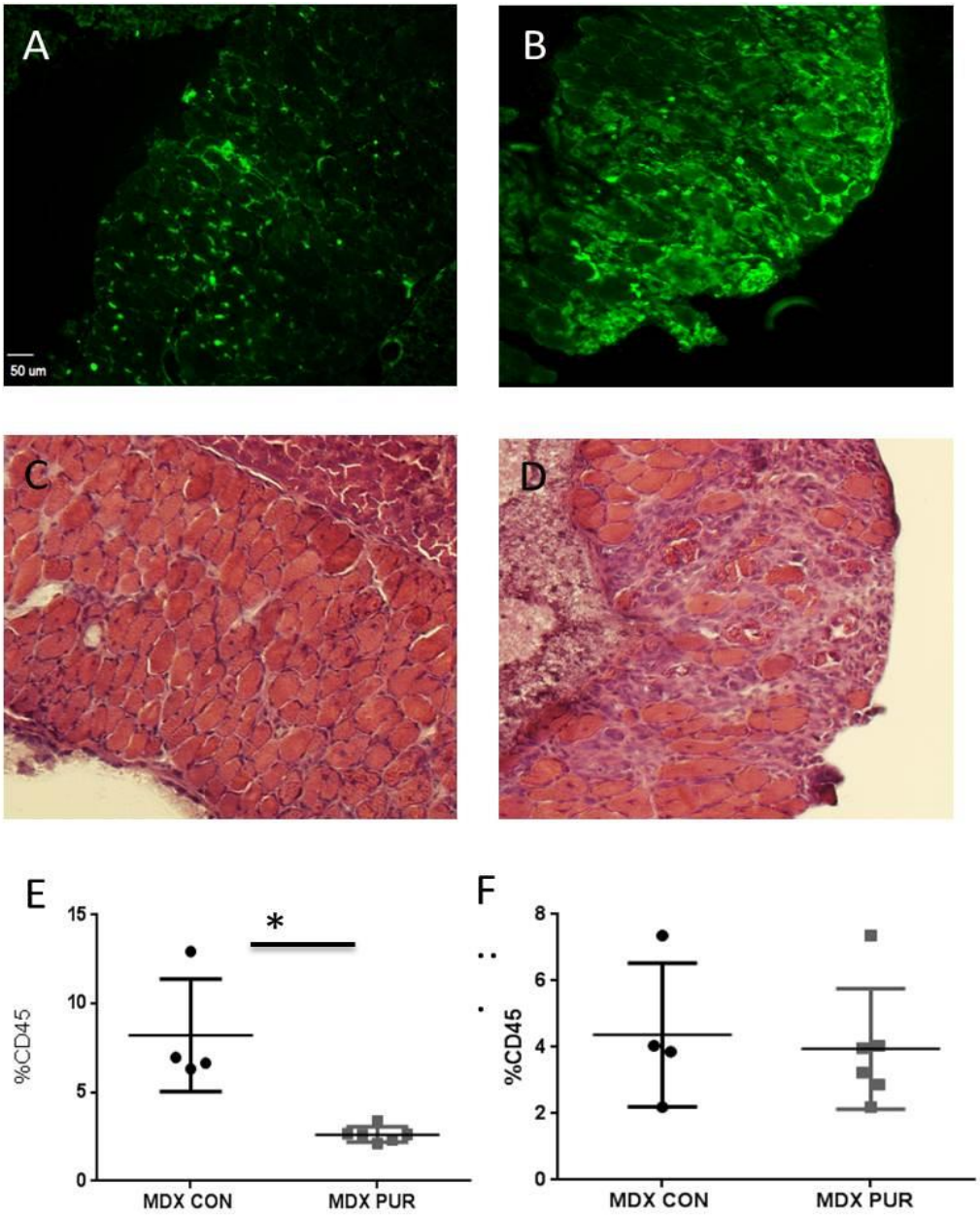



**Figure 7.** **A**.CD45 (green) Immunostaining of diaphragm from 9 week old *mdx* mouse treated with purmorphamine from 8 to 9 weeks of age. **B**. Control *mdx* mouse, 9 weeks of age, CD45 immunostaining. **C.** Hematoxylin and Eosin sections of same tissue as A **D.** Hematoxylin and Eosin sections of same tissue as B. **E**. CD45 staining quantified at the total positive area measurement relative to total tissue area. Treatment with purmorphamine for Hh activation

significantly reduces general immune response in diaphragm tissue. **F**. The effects of Hh signaling activation is not seen in the quadriceps of the same mice. Control N=5, Treated N=6 * P<0.05

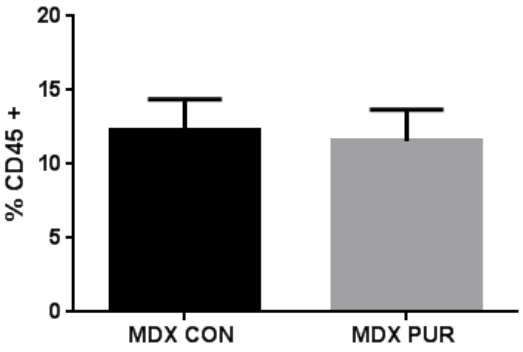
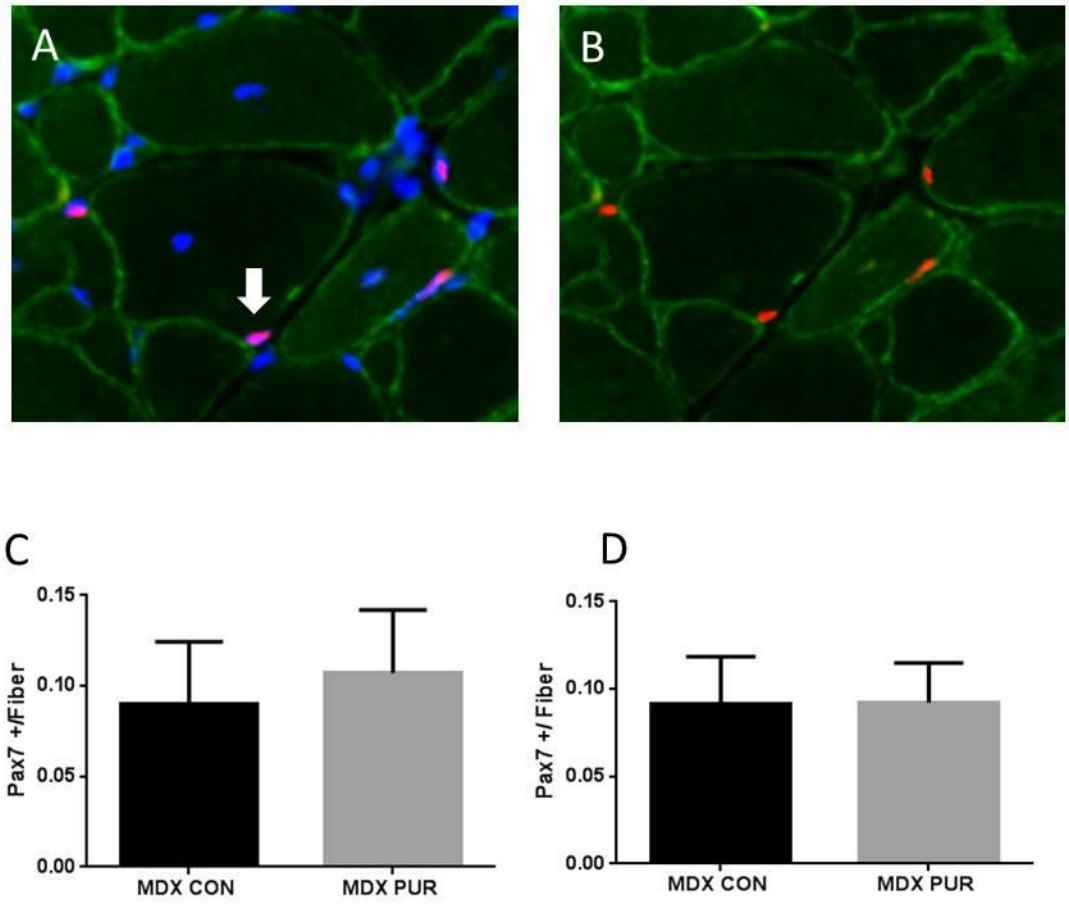



**Figure 8.** Mice treated for 2 months with purmorphamine showed no difference in general inflammation by CD45 staining in the diaphragm.

## Satellite Cells

Having previously seen an upward trend for the number of myofibers per area of tissue (Figure 5) we wanted to better understand the cause of this observation. The change could be due to decreased fibrosis or improved regeneration which is what led me to look at satellite cells. Satellite cells are known to be depleted in DMD and the *mdx* mouse. In order to determine if Hh signaling alters the amount of satellite cells in muscle, tissue sections were stained for Pax7, a marker of satellite cells, which was normalized to the total number of fibers in the tissue section for entire muscle, **Figure 9**. There was no difference between control and treated mice in the number of satellite cells in the diaphragm or the quadriceps.

**Figure 9. A.** Pax7 (red) positive satellite cells overlapped with Dapi(blue) to show satellite cells (purple) in quadriceps tissue of 9 week old mice. **B**. Pax7 positive cells shown in red in quadriceps tissue of 9 week old mice. **C.** No difference in diaphragm for Pax7 positive satellite cells after one week of Hh activation **D.** No difference in quadriceps for Pax7 positive cells after one week of Hh activation. Control N=5, Treated N=6




**Satellite Cell Differentiation**

We next looked at whether Hh activation might improve satellite cell differentiation rather than activation. In order to quantify the effects that activated Hh signaling may have on regeneration, tissues were stained with eMHC, a marker of regenerating fibers and laminin, a marker of the basal lamina. On entire tissue sections, fibers were classified in three categories. The fiber could be labeled as "full regeneration" which means that the process was near completion and the entire basal lamina scaffold had been re-filled with a moyfiber; labeled as "normal regeneration", where the fiber is still undergoing regeneration as evidenced by the presence of multiple small caliber myotubes inside the original basal lamina scaffold; and "branching" which denotes fibers

where myotubes have stopped growing prematurely and have started to deposit their own basal lamina thus generating branched fibers. These classifications can be seen in **Figure 10.**

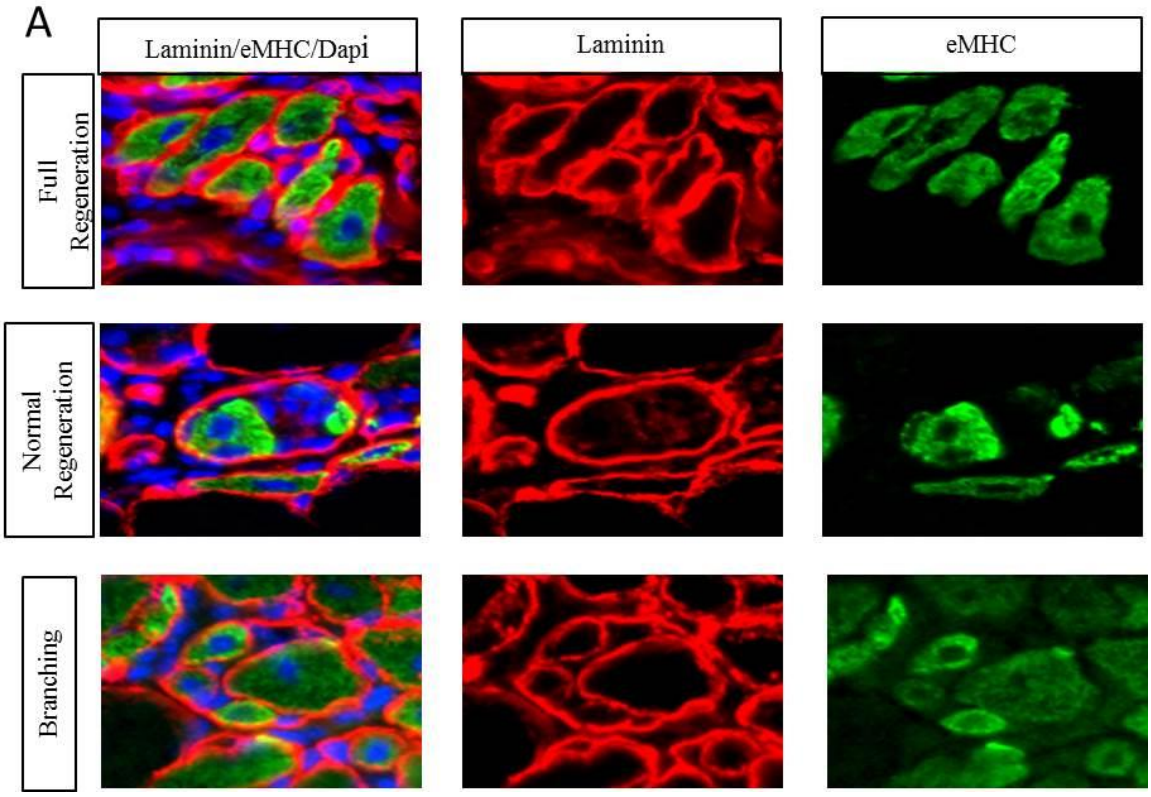



**Figure 10 A.** Immunoflorescent staining for eMHC (green) to observe regeneration in treated *mdx* mice. Sections from mice treated with purmorphamine for 1 week from 8 weeks of age to 4 weeks of age. Full regeneration shows fibers in which the satellite cells have fully fused and differentiated back to the original fiber size with no defects. Normal regeneration denotes a fiber that is actively regenerating. Nuclei (blue) can be seen in large quantities inside the cell and will continue to fuse and express eMHC or will be impaired and begin to branch. Branching fibers are those which have an impaired repair process and did no fuse properly. Rather, laminin (red) was deposited around fused areas and forms individual fibers that branch from each other rather than fusing into one larger fiber.

In order to determine the extent of branching in the muscle I compared the different categories of regeneration between muscle. The eMHC positive fibers were classified into the three groups of "full regeneration", "normal regeneration" and "branching". The total in each category was normalized to the total number of eMHC positive fibers in the tissue. Comparisons were made between quadriceps and diaphragms of 1 week treated mice, **Figure 11**.

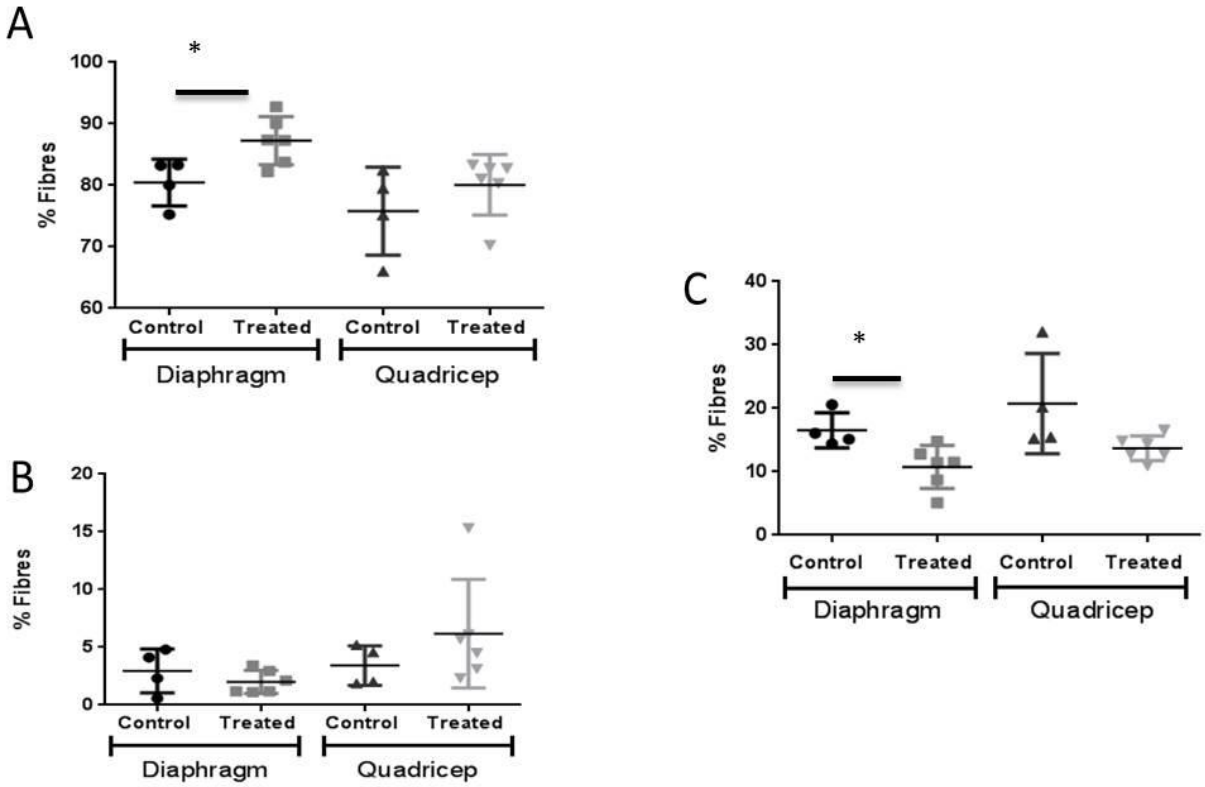
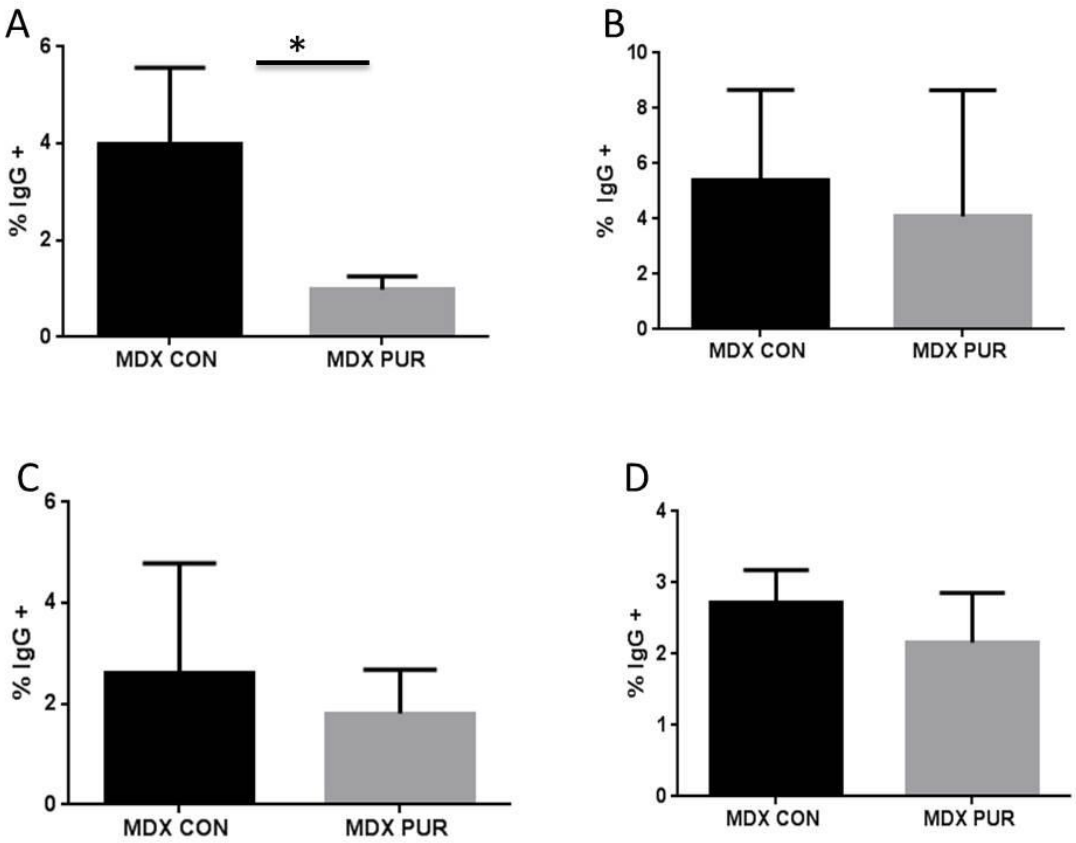



**Figure 11. A.** In mice treated for just one week, there was a significant amount of fibers that were able to regenerate fully with no impairments. **B.** There is no difference in the number of fibers in the initial state of regeneration **C.** The number of fibers that become branched due to an impaired regeneration process is reduced. Note: For B-D the individual samples are denoted on the graphs rather than an average for the group to show distribution in each category. Control N=5, Treated N=6 *P<0.05

**Fiber Damage**

With improved regeneration and a decrease in branched fibers it would be expected to see a decrease in damage in the muscle. Since the branched fibers are more prone to damage we wanted to see if decreasing their presence in muscle would decrease the damage. In order to measure this outcome I assessed damage sustained by the muscle using an IgG stain against mouse. Fibers which show staining have been damaged and the number of damaged fibers relative to the total number of fibers was measured. Mice treated for two months showed a significant decrease in damage in the diaphragm but this improvement was not seen in the quadriceps. There was no decrease of damage in mice treated for one week in both muscle types, **Figure 12**.



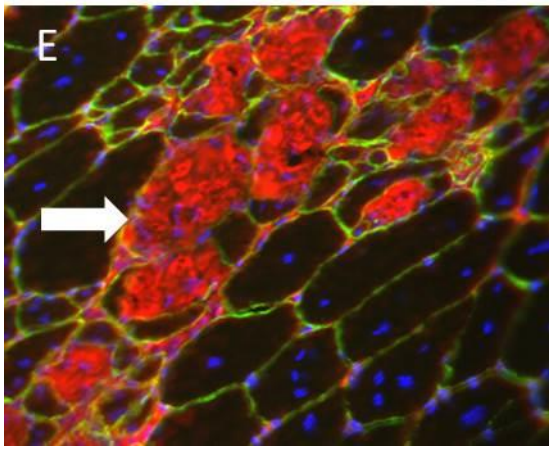



**Figure 12. A.** Mice treated for two months with purmorphamine showed a significant decrease in IgG positive (necrotic) fibers, out of the total number of fibers in an entire tissue section in the diaphragm. **B.** In the same mice treated for 2 months there was no change seen in the quadriceps **C.** Diaphragm of mice treated for 1 week does not show a significant change in IgG positive fibers. **D.** The quadriceps of 1 week treated mice does not show a change in IgG positive fibers. **E.** Image in Quadricep muscle of an IgG positive fiber to denote damage. The white arrow denotes a positive fiber. * $P<0.05$. For **A** and **B,** Control N=5, Treated N=3. For **C** and **D C**ontrol N=4, Treated N=6. Staining of diaphragms from mice treated for 2 months completed by Sarah Tuthill.

## Discussion

Current research is aimed at better understanding the mechanisms and pathways involved in DMD. With no cure available, finding a treatment that can alleviate the secondary effects of the loss of dystrophin is desired. Members of our lab discovered that myogenic differentiation of interstitial stem cells is dependent upon active Hedgehog signalling which is inhibited in the dystrophic muscle environment. It is important to define the broader role of this pathway in the progression of DMD.

When aiming to better understand the Hh pathway it is important to look closely at the regeneration process as a whole in muscle.

It is interesting to note the difference in the response to treatment between the quadriceps and the diaphragm muscles. There is consistently an effect seen in the diaphragm but not in the quadriceps compared to controls. The two muscles progress differently in the disease with the diaphragm being more severely affected. Because the quadriceps are not as severe there may be less room for improvement (Haslett et al., 2005). These contrasts may be due to different alterations in Hh signalling in each muscle. Understanding this outcome is important to assess how the pathway affects muscle. In order to better

understand these contrasts, we are quantifying the decrease in Hh activity between diaphragm and quadriceps at different ages in *mdx* mice. Testing a range of time points will potentially show an altered activation in the diaphragm compared to the quadriceps. In addition, Hh ligands could be identified in muscle sections to see if the quantity or location is different in diaphragm compared to quadriceps muscle.

**Inflammation**

The inflammatory response plays a role in altering regeneration and fibrosis deposition so understanding how Hh signalling alters the inflammatory response is important. I found that inflammation was decrease in the diaphragms of mice treated for one week. However, a better understanding the characteristics of this decrease are important. The presence of M1 and M2 macrophages can be studied as they are primary players in the immune response. Macrophages can be beneficial and promote regeneration, but in some cases they can be cytotoxic and can damage the muscle (Nguyen and Tidball, 2003), which is what can be seen in the *mdx* model. The decrease seen in the immune response after one week may be explained by a mechanism which controls this response and prevents negative effects from the macrophages. In order to test this hypothesis, cytokines involved in M1 and M2 macrophage activation can be tested at different time points in the repair process. It has been found that macrophages help to increase the proliferation of satellite cells but that they may prevent the differentiation of these cells (Merly et al., 1999; Tidball and Villalta, 2010) . In order to understand if there is an alteration in dystrophic muscle I am comparing the presence of macrophages in tissue. To assess the changes in M1 and M2 macrophages, a co-labeling is being done with F4/80 which is a marker of M1 macrophages and CD206, a marker for M2 macrophages. While the diaphragms have not yet been characterized, I have compared the differences in the quadriceps. Although there is not a significant difference in macrophage types, it is important to still look at the diaphragms as they continuously turn out differently than the quadriceps due to differences in disease progression. Our findings, along with those mentioned, may represent a connection between the decreased

inflammatory response seen and the improved regenerative properties of the treated muscle.

**Satellite Cells**

When we saw a decrease in fibrosis in mice treated for two months we wanted to better understand the cause of this reduction. Inflammation, along with alterations in the ability of satellite cells to differentiate can affect fibrosis. Using the Pax7 staining to show satellite cells there was no difference seen between treated mice and control mice. Although there is no effect seen, this assay does not distinguish between those that go through differentiation successfully. Therefore, although the presence of satellite cells is the same, those in the *mdx* mouse may not be able to differentiate as successfully which leads to branched fibers. Or, there could be no differentiation at all due to an impaired process. It would be important to further study the fate of the activated cells to better understand the specific processes that are occurring.

If regeneration is improved and there are less branched fibers, which are more easily damaged, it would be expected that there is less damage in the muscle. In order to see if this assumption was correct, I conducted an IgG stain to denote necrotic fibers. There was no significant difference between treated and control mice after just one week of treatment. What may be suggested here is due to the early stage in the disease, treated mice have not had a chance to catch up on preventing damage compared to controls. New fibers are beginning to regenerate in both tissues so there may not have been enough time between regeneration and tissue collection for a significant number of fibers to become damaged. In order to see if this assumption was correct and that the treatments were preventing damage I looked at an IgG staining in the mice treated for 2 months. The diaphragms, which were stained by Sarah Tuthill, showed a significant reduction in damage. The quadriceps however, did not see the same changes. The reduction in the diaphragm may be due to the decrease of branching in the muscle which lessens contractile damage. Although the Hh signalling activation does not eliminate the damage, the decrease can reduce inflammation and further need for extensive regeneration. This

outcome would lead to a decrease in the deposition of fat and fibrosis and improve the muscle environment.

**Fibrosis**

If the muscle is damaged and is not able to properly repair, fibrotic tissue is deposited. Fibrosis within muscle can impair function and can decrease the amount of muscle fibers present in a tissue. When regeneration fails, cells can begin to release pro-fibrotic signals and cause the deposition of fibrosis. While this is normal in muscle as aging occurs it is exaggerated in DMD. It would be expected that since the inflammation is more controlled and regeneration is improving that there would be less fibrosis deposition which is what we see in the diaphragm. My findings support the notion that the treatment is not impairing regeneration, as suggested by less centrally nucleated fibers after long term treatment. Rather, there is less damage, improved myofiber regeneration, and less deposition of fibrotic tissue.

**Long Term Outcome**

Since we have seen alterations in inflammation, regeneration, damage and fibrosis in short and long term treatments we wanted to see the effect on functional ability in the mice. In order to measure changes, Dr. Christopher Penton conducted an exercise induced fatigue test which monitors their movement pre and post exercise. . We found that just after one month of treatment, mice had a significant reduction in fatigue compared to controls. This result was carried out after two months of treatment, **Figure 13.**

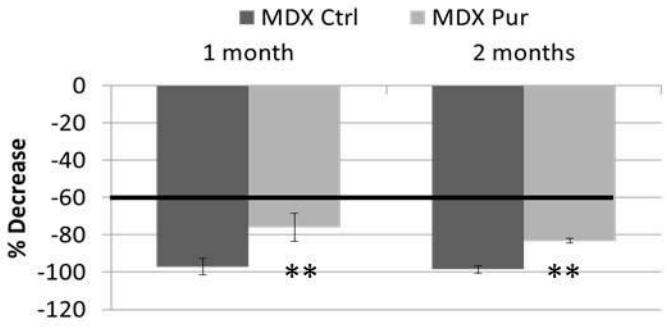



**Figure 13.** A behavioural test to observe resistance to fatigue was done by Dr. Christopher Penton in mice treated for two months with Purmorphamine. Mice were monitored in an open field activity cage for 1 hour pre and post

exercise. Mice were run for 10 minutes at 10m/min on a 15 degree decline. This figure shows decrease in rearing in mice after exercise. The black line denotes the decrease seen in WT mice. **P<0.01

While utilizing purmorphamine as an Hh agonist improves pathology, using a genetic model to study the effects is also of interest. In order to observe if the same changes would be present we are working on characterizing the same aspects in a genetic model known as the patched-1 mouse. We have back crossed some of the mice to the 5cv and WT genotypes and are seeing if the model can reproduce the same results with activation throughout life. The patched mouse does not actually activate hedgehog signaling, rather it eliminates a copy of patched and therefore allows smoothened to be activated more readily even at lower concentrations of ligand. If similar results are seen through a genetic model, this research could potentially be applied via gene therapy.

All of the effects that lead to the pathology in DMD have yet to be understood. The Hh pathway is not commonly studied or well understood in muscle, and dystrophic muscle in particular. This study has shown that the pathway can positively alter pathology so it is of importance to characterize the effects. While it is beyond the scope of this thesis it is important to better understand the mechanisms involved in the pathway. If Hh signaling only alters one of the areas affected but it has a cascading effect to other secondary effects it would be important to understand the way this occurs. More knowledge on the pathway functions can help in better understanding DMD along with providing the opportunity to study potential therapeutic treatments.

**Abbreviations**

Duchenne Muscular Dystrophy: DMD

Hedgehog: Hh

Extra Cellular Matrix: ECM

Dystrophin Glycoprotein Complex: DGC

Intraperitoneal: IP

Wild Type: WT

Smoothened: Smo

Patched-1: Ptch

Transforming growth factor beta: TGF-β

Paired Box 7: Pax7

Inducible Nitric Oxide Synthase: iNOS

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